Hydrolysis of Phthalyl Amino Acid Esters of Fluoroscein in the Presence of Leucine Aminopeptidase[1] (36421)

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Knowledge of the mechanisms of action, and diagnosis of persistent and latent viral infection is becoming increasingly important in the study of oncology, infection resistance, and virus replication. If vaccine production is an objective, diagnosis of latent infection is particularly important.

Bouillant and Daniel (1, 2) noted that KB cells, after latent infection with Myxovirus parainfluenza type 3 displayed strong leucine aminopeptidase (LAP) activity. Thus detection of elevated levels of aminopeptidase activity may present a reliable method for determining Myxovirus parainfluenza type 3 infection in these cells.

The method used by Bouillant and Daniel for detecting LAP activity depends upon the enzymatic hydrolysis of L-leucyl-beta-napthylamide. This method, although widely used, is tedious and time consuming. A simpler, more rapid, and more widely applicable procedure would permit more extensive studies of the activity of this enzyme for diagnostic and epidemiological purposes.

Rotman and Papermaster (3), Guilbault *et al.* (4–8) and others (9–11) have developed fluorescein esters as fluorogenic substrates for various enzymes including lipase, esterase, and sulfatase. Fluorecein esters are particularly advantageous for diagnostic purposes, since upon hydrolysis the liberated fluorescein emits a bright green fluorescence detectable by eye at one part in forty million. Thus fluorescein esters possessing amino acid or peptide moieties may prove suitable fluorogenic substrates for proteolytic enzymes such as LAP.

Accordingly, the following phthalyl amino acid esters of fluorescein were synthesized:

I. *l, l-bis* (α-isobutyl-1, 3-dioxo-2-isoindolineacetyl)-fluorescein
II. *l, l-bis* (α-butyl-1, 3-dioxo-2-isoindolineacetyl)-fluorescein
III. *l, l-bis* (α-isopropyl-1, 3-dioxo-2-isoindolelineacetyl)-fluorescein
IV. *d, d-bis* (α-isobutyl-1, 3-dioxo-2-isoindolineacetyl)-fluorescein
V. *l, l-bis* (α-propyl-1, 3-dioxo-2-isoindolineacetyl)-fluorescein
VI. *l, l-bis* (α-methyl-1, 3-dioxo-2-isoindolineacetyl)-fluorescein
VII. *d, d-bis* (α-isopropyl-1, 3-dioxo-2-isoindolineacetyl)-fluorescein

To determine substrate specificity, the synthesized esters were tested *in vitro* with LAP and various enzymes such as lipase, chymotrypsin, trypsin, acetylcholinesterase, and butyryl cholinesterase. *Methods*: The methods of preparation of the fluorogenic ester substrates are analogous to those developed by Meyer-Bertenrath *et al.* (11) in synthesizing fatty acid esters of fluorescein from the corresponding acyl chlorides. The precursor, phthalyl amino acids, were either obtained

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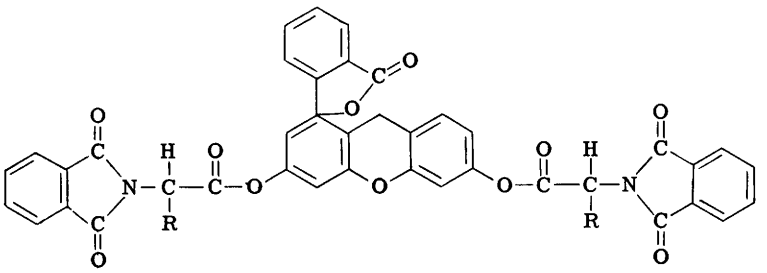
TABLE I. Analytical Data Phthalyl Amino Acid Esters of Fluorescein.



Compound number	R	Melting point °	$[\alpha]_D$ 25°[a]	Molecular formula	Calculated C	Calculated H	Calculated N	Found C	Found H	Found N
I	$CH_2CH(CH_3)_2$	266–268	(—) 91.6°	$C_{48}H_{38}N_2O_{11}$	70.41	4.68	3.42	70.25	4.72	3.32
II	$CH_2(CH_2)_2CH_3$	220–223	(—) 106.9°	$C_{48}H_{38}N_2O_{11}$	70.41	4.68	3.42	70.39	4.82	3.42
III	$CH(CH_3)_2$	235–238	(—) 134.2°	$C_{46}H_{34}N_2O_{11}$	69.89	4.30	3.54	69.85	4.45	3.50
IV	$CH_2CH(CH_3)_2$	264–267	(+) 91.8°	$C_{48}H_{38}N_2O_{11}$	70.41	4.68	3.42	70.32	4.72	3.30
V	$CH_2CH_2CH_3$	216–219	(—) 105.6°	$C_{46}H_{34}N_2O_{11}$	69.89	4.30	3.54	70.04	4.38	3.41
VI	CH_3	195–198	(—) 116.2°	$C_{42}H_{26}N_2O_{11}$	68.66	3.57	3.81	68.77	3.81	3.75
VII	$CH(CH_3)_2$	234–237	(+) 134.0°	$C_{46}H_{34}N_2O_{11}$	69.89	4.30	3.54	69.72	4.38	3.41

[a] 40 mg/ml in $CHCl_3$.

commercially (Mann Research Laboratories) or synthesized using procedures developed by Nefkens (12). They were converted to the corresponding phthalyl amino acyl chlorides using thionyl chloride in the usual manner.

Enzymes: Leucine aminopeptidase (LAP) was obtained from pig kidney (Mann Research Laboratories, activity 122 units/mg protein, 70% ammonium sulfate suspension with Tris buffer and magnesium chloride, contains 5.0 mg/ml).

Lipase was obtained from porcine pancreas (Mann Research Laboratories, 1.2 lipase units/mg).

Chymotrypsin, alpha, was obtained from beef pancreas (Nutritional Biochemicals Company, 45 units/mg BTEE, or 10,000 units/mg ATEE at 25°).

Trypsin was obtained from beef pancreas (Nutritional Biochemicals Company 3000 NF units or 180 TAME units/mg, chymotrypsin contamination *ca.* 0.5%).

Butyryl colinesterase (horse serum) was obtained from nonerythrocytic origins (Mann Research Laboratories, 6 units/mg acetylcholine at 25°).

Acetylcholinesterase was obtained from bovine erythrocytes (Mann Research Laboratories, 100 units/mg).

Tris Buffer. Tris (hydroxymethyl) aminomethane, 0.11 *M* was prepared by dissolving the appropriate amount of Sigma 7–9 buffer (Sigma Chemical Company) in distilled water. Concentrated hydrochloric acid was used to adjust the pH to the desired value (7.2).

Apparatus. All fluorescence measurements were made with a Turner Model 111 spectrofluorometer. A constant temperature of 25° was maintained with a thermo-electric cooler. The excitation wavelength was 490 mμ and the emission wavelength was 520 mμ.

Procedure. Each substrate was weighed and dissolved in appropriate amounts of acetone to yield various concentrations (0.5, 0.75, 1.0, 1.12, and 2.0 mg/ml). A 0.025-ml portion of this solution was added to 2.5 ml of Tris–HCl buffer solution (pH 7.2) to prepare the blank. A stock solution composed of 0.54 ml of Tris–HCl buffer and 0.41 ml of commerical LAP ammonium sulfate suspension was prepared. Stock solutions of the oth-

TABLE II. Effect of Various Enzymes on Hydrolysis of Fluorescein Esters (ΔF/min/25°/pH 7.2.[a]

Substrate	Blank rate	LAP	Chymo-trypsin	Trypsin	Lipase	Butyryl Cholin-esterase	Acetyl-cholin-esterase
I	2.00	10.25	1.40	0.10	1.77	1.08	0.03
II	0.36	3.64	2.20	0.02	1.20	(—)0.10[c]	0.21
III	0.32	0.86	1.09	0.26	0.22	0.00	(—)0.13[c]
IV	1.73	6.33	4.00	0.47	2.65	0.04	(—)0.14[c]
V	2.70	11.00	3.00	0.06	1.70	0.52	0.28
VI	3.20	20.50	8.40	0.30	9.43	4.08	4.00
VII	0.57	1.87	0.80	0.20	0.70	0.28	0.00
Fluorescein diacetate (FDA)[b]	2.80	96.00	27.14	1.90	28.66	9.28	8.40
Fluorescein dipropionate (FDP)[b]	1.20	96.00	46.00	0.85	10.00	14.00	10.86
Fluorescein dibutyrate (FDB)[b]	1.40	56.00	57.33	(—)1.00[c]	92.00	42.00	10.50
Fluorescein dioctonoate (FDO)[b]	0.07	0.77	50.00	0.03	9.04	0.34	0.96
Fluorescein didodeconoate (FDD)[b]	0.23	9.70	0.10	(—)0.13[c]	1.42	0.36	0.52

[a] All enzyme concentrations are 0.078 mg/ml. All substrate concentrations are 0.038 mg/ml.

[b] Nutritional Biochemicals Corporation.

[c] The blank rate of hydrolysis exceeds the enzymatic rate by the amount indicated.

er enzymes were prepared by adding 2.0 mg of enzyme to 1.0 ml of Tris–HCl buffer solution at pH 7.2. A 0.1-ml portion of the enzyme stock solution was added to 2.5 ml of Tris-HCl buffer solution and 0.025 ml of the acetone substrate solution. The rate of increasing fluorescence of the blank and the enzyme containing solution was recorded. Enzyme activity can be measured by determining the rate of increase in fluorescence (ΔF/min, blank rate subtracted) for each preparation (Table II).

Results. The phthalyl amino acid esters of fluorescein are colorless, crystalline, high-melting and stable (Table I).

The data in Table II indicate the differing rates of hydrolysis of each substrate with each enzyme. In addition, the hydrolysis rates of five aliphatic acid esters of fluorescein (fluorescein diacetate (FDA), dipropionate (FDP), dibutyrate (FDB), dioctonoate (FDO), and didodecanoate (FDD)), were also tested. As is seen, the differences in rates of ester cleavage by the various enzymes are in many cases quite large. The enzymatic hydrolysis rates, in general, are rapid in the case of LAP, lipase, and chymotrypsin and much less with both esterases and trypsin. Since LAP has a molecular weight approximately ten times greater than either chymotrypsin, trypsin, or lipase, the hydrolysis rate differences between LAP and these three enzymes are much greater if considered on a molar basis (15). Close linearity of response is observed during the first ten minute period of hydrolysis in all cases.

The Michaelis constant (K_m) values of the substrates for LAP were determined (Table III). It should be noted that the affinities of all these esters for LAP are greater than that of leucine amide (K_m 5.21×10^{-3}). Also, in Table III, the minimum detactable amounts

TABLE III. Comparison of K_m Values of Various Substrates for LAP.

Substrate	K_m	Minimum detectable enzyme concentration (μg/ml)
I	1.13×10^{-5}	8.0
II	2.17×10^{-5}	8.0
III	2.94×10^{-5}	6.0
IV	1.47×10^{-5}	5.0
V	1.05×10^{-5}	7.0
VI	9.09×10^{-6}	2.0
VII	2.05×10^{-5}	1.0
FDA	2.86×10^{-5}	0.8
FDP	1.37×10^{-5}	0.2
FDB	1.98×10^{-5}	1.0
FDO	1.89×10^{-5}	1.0
FDD	1.00×10^{-5}	8.0

of enzymes for the various substrates are listed. These are the minimum concentrations of enzyme required to obtain a hydrolysis rate twice that of the blank rate.

Discussion. Fluorescein esters of fatty acids have been shown to be metabolized by living cells (3, 9, 10, 13). The rate of hydrolysis can be altered by vaccinia virus infection (14). The present results indicate that the new substrates I, V, and VI, which have high affinity and a certain amount of specificity for LAP may possibly serve as useful diagnostic reagents for latent parainfluenza virus infection (1, 2). Some of the substrates reported herein are currently being tested for detecting certain virus infections in various cell lines.

Summary. Phthalyl amino acid esters of fluorescein were synthesized and characterized. They were tested (*in vitro*) for use as possible fluorogenic substrates for LAP. The rates of enzymatic hydrolysis of these compounds were compared with those of some commercially available fluorescein esters.

1. Bouillant, A., and Daniel, P., Can. J. Microbiol. **14**, 971 (1968)

2. Bouillant, A., and Daniel, P., Can. J. Microbiol. **14**, 975 (1968).

3. Rotman, B., and Papermaster, B. W., Proc. Nat. Acad. Sci. U.S.A. **55**, 134 (1966).

4. Guilbault, G. G., and Kramer, D. M., Anal. Biochem. **14**, 28 (1960).

5. Guilbault, G. G., and Kramer, D. M., Anal. Chem. **36**, 409 (1969).

6. Guilbault, G. G., and Hilserman, J., Anal. Chem. **41**, 2006 (1969).

7. Guilbault, G. G., "Enzyme Methods of Analysis," Pergamon Press, London, New York (1963).

8. Guilbault, G. G., Anal. Chem. **40**, 459R (1968).

9. Rotman, B., Zderic, J. A., and Edelstein, M., Proc. Nat. Acad. Sci., U.S.A. **50**, 1 (1963).

10. Medzon, E. L., and Brady, M. L., J. Bacteriol. **97**, 402 (1969).

11. Meyer-Bertenrath, J. E., Hoppe-Seyler's Z. Physiol. Chem. **349**, 728 (1968).

12. Nefkens, G. H. L., Nature (London) **185**, 309 (1960).

13. Medzon, E. L., and Gedies, A., Can. J. Microbiol. **17**, 651 (1971).

14. Medzon, E. L., and Faught, J., Abstracts, Canadian Society of Microbiologists, 21st meeting, London, Ont., June 1971.

15. Barman, T. E., "Enzyme Handbook," Vol. II. Springer-Verlag, New York (1969).

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